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1) J Chromatogr 1990 Feb 23;525(2):297-306
Purification of urokinase by combined cation exchanger and affinity chromatographic cartridges.
Hou KC, Zaniewski R.

2) Purification of high-molecular-weight and low-molecular-weight urokinase and kinetic study
Sun, Tian-Xiao; Wang, Hong-Mei; Xu, Chang-Fa
Shengwu Huaxue Zazhi (1997), 13(3), 344-349

3) Isolation, purification and comparative studies of certain properties of high- and low-molecular-weight urokinases of human urine.
Sun, Leqin; Zhang, Hongzu; Zhu, Dexu
Shengwu Huaxue Yu Shengwu Wuli Xuebao (1984), 16(3), 303-6

4) J Biochem (Tokyo) 1981 Jul;90(1):225-32
A comparative study of high molecular weight urokinase and low molecular weight urokinase.
Nobuhara M, Sakamaki M, Ohnishi H, Suzuki Y.

5) Enzyme 1981;26(4):221-4
Kinetic studies of three different molecular forms of urokinase for the activation of native human plasminogen.
Toki N, Takasugi S, Sumi H.

6) Thromb Haemost 1983 Apr 28;49(2):91-5
Purification of high molecular weight urokinase from human urine and comparative study of two active forms of urokinase.
Shibatani T, Kakimoto T, Chibata I.

7) Thromb Haemost 1982 Jun 28;47(3):197-202
Rapid isolation of high molecular weight urokinase from native human urine.
Huber K, Kirchheimer J, Binder BR. 6788545

8) Chem Pharm Bull (Tokyo) 1981 Feb;29(2):463-71
Comparative studies on two active enzyme forms of human urinary urokinase. I. Purification by serial column chromatography and homogeneity analyses of molecular weight and isoelectric point.
Miwa N, Takayanagi H, Suzuki A.

Thank you,
David J. Steadman
Art Unit 1652
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308-3934

son, S.; Westman, S.: Mast cell con-
acid metabolism in the epididymal
ese mice. Acta physiol. scand. *58:*
3).
ı, E.: Methods for determination of
:e in man and animals. J. Lipid. Res.
)68).
cerolipid biosynthesis in rat adipose
n. J. *170:* 153–160 (1978).
ıpiro, D.; Fallon, H.J.: Triacylglyce-
is in adipose tissue of the obese-
c mouse. Biochem. J. *158:* 327–334

coff, M.A.; Bell, R.M.: Triacylglyc-
in isolated fat cells. An effect of insu-
ımal fatty coenzyme A ligase activi-
:m. *251:* 1488–1492 (1976).
ty acid synthesis in adipose tissue
'itiated water. Biochemistry *7:* 3708–

ıction of insulin effector system of
cells by proteolytic enzymes. J. biol.
'77–5784 (1969).
:s, A.B.: Metabolism of isolated fat
ılar inhibitory actions of phospholi-
dium perfringens α toxin) and lipo-
:s and theophilline. J. biol. Chem.
(1966).
owski, M.J.; Segal, R.: Effect of insu-
:llular character of rat adipose tissue.
13: 616–623 (1972).
erti, K.G.G.M.; Williamson, D.H.:
tozotocin on carbohydrate and lipid
ı the rat. Endocrinology *89:* 827–834

aggerson, E.D.: Interactions of insu-
aline with glycerolphosphate acyla-
in fat-cells from rat. FEBS Lett. *64:*

herjee, C.; Jungas, R.L.: Studies on
n of activation of adipose tissue py-
ogenase by insulin. J. biol. Chem.
)73).

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ıte of Technology, 3325 W. New
e, Melbourne, FL 32901 (USA)

Enzyme *26:* 221–224 (1981)

Kinetic Studies of Three Different Molecular Forms of Urokinase for the Activation of Native Human Plasminogen

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Key Words. Urokinase · Glu-PG · Lys-PG · Michaelis constant · Catalytic constant

Abstract. The kinetic parameters of three different molecular forms of urokinase (UK) for the activation of native Glu-plasminogen were compared. The apparent Michaelis constant ($K_{m,app.}$) of each UK was almost of the same order of magnitude (31–38 μ*M*), but the catalytic constants (k_c) were observed to be different: UK_h (high molecular weight form, molecular weight 53,000), 2.4 ± 0.2 s^{-1}; UK_l (low molecular weight form, molecular weight 33,000), 0.83 ± 0.10 s^{-1}, and UK_t (trypsin-digested form, molecular weight 36,000), 0.91 ± 0.18 s^{-1}. The overall second order rate constant, k_c/K_m calculated for UK_h was 7.7 × 10^4 M^{-1} s^{-1}, higher than for UK_l (2.2 × 10^4 M^{-1} s^{-1}) or UK_t (2.4 × 10^4 M^{-1} s^{-1}), indicating the possibility of a much higher degree of enzymatic specificity and efficiency.

Introduction

Christensen and Müllertz [1] and *Christensen* [2] compared the kinetics of urokinase (UK) in the activation of native Glu-plasminogen (Glu-PG) and its partially degraded form (Lys-PG), and concluded that the binding characteristics (the Michaelis constant, K_m) are identical for the two molecules, but their catalytic rates (k_c) are significantly different (Lys-PG ≫ Glu-PG). Recently, a few authors have demonstrated the difference in thrombolytic effect and PG activation activity by different UK molecules using the fibrin plate method [3, 4], Chandler's loop method [3], and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis [5].

In this report, the kinetic parameters of three different molecular forms of UK for the activation of native Glu-PG were compared in purified systems.

Materials and Methods

All materials used were of reagent grade quality. The determination methods of protein and UK activity, and immunoelectrophoresis were the same as described previously [6, 7]. The molecular weight of UK was determined using SDS polyacrylamide gel electrophoresis according to the method of *Weber and Osborn* [8].

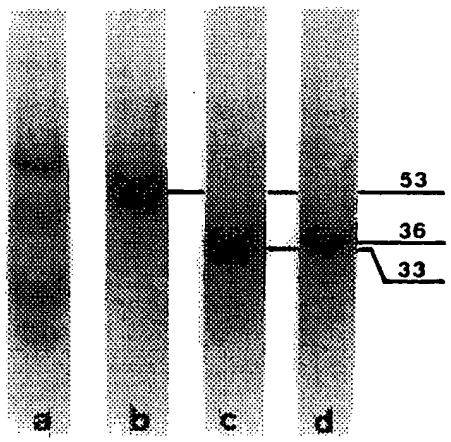

Fig. 1. SDS polyacrylamide gel electrophoresis of purified UK. Sample diluent consisted of 6.0 mol/l urea, 1% SDS and 0.01 mol/l sodium phosphate buffer at pH 7.0. a = Reference proteins of bovine serum albumin, egg albumin, chymotrypsinogen A and cytochrom c; b = UK_h; c = UK_l; d = UK_t, respectively. The numbers on the right indicate the molecular weight in thousands.

Urokinase. Three different molecular forms were prepared: (1) UK_h, molecular weight 53,000, which is assumed to be the native UK form [6, 9–13], was purified from a commercial preparation (The Green Cross Co.) by affinity chromatography on [N^{α}-(ε-aminocaproyl)-*DL*-homoarginine hexylester]-Sepharose column followed by Sephadex G-100 gel filtration, as described previously [6]. (2) UK_l, molecular weight 33,000, which is the modified UK form, was also purified by the same method as UK_h from a commercial preparation (Mochida Pharmaceutical Co.) as described previously [6]. (3) UK_t, molecular weight 36,000, which is a trypsin-digested form, was prepared from UK_h by a method based on that of *Lesuk* et al. [11]. 0.2 ml of trypsin (Sigma Chemical Co.: 0.86 μg/ml of 0.05 mol/l Tris-HCl, 0.01 mol/l $CaCl_2$, pH 8.0) was added to 1.0 ml of UK_h (2.0 mg/ml of 0.05 mol/l Tris-HCl, 0.1 mol/l NaCl, 0.01 mol/l $CaCl_2$, pH 8.0). After the incubation of this mixture for 5 h at room temperature, UK_t was separated by Sephadex G-100 (Superfine) gel filtration (column: 1.0 × 160 cm). Elution was performed with 0.15 mol/l ammonium bicarbonate, 0.2 mol/l NaCl, and with that portion which corresponded to the active peak of the partition coefficient K_{av} [14] 0.39 was collected. Purified preparations were concentrated by ultrafiltration (Collodion-bags, SM 13,200) in the presence of mannitol (1.0%) and lyophilized. They were observed to be homogeneous when subjected to immunoelectrophoresis [6]. In SDS polyacrylamide gel electrophoresis, UK_l and UK_t showed a single protein band, but UK_h showed one main and one minor band (m.w. approx. 100,000, possibly the dimer form of UK_h or of another contaminating protein) (fig. 1). The specific activities determined by the fibrin plate method [6] were: UK_h 87,000; UK_l 79,900, and UK_t 101,000 international units (IU)/mg of protein, respectively.

Human Glu-PG. Purification and determination methods were the same as described previously [1, 2] (>96% Lys-PG as NH_2-terminal amino acid determined qualitatively by the method of *Gros and Labouesse* [15]).

Kinetics. The kinetic parameters were determined as described by *Christensen and Müllertz* [1] and *Christensen* [2]. In a solution containing UK, Glu-PG and α-N-benzoyl-*L*-arginine ethylester (Bz-Arg-OEt) (Sigma Chemical Co.), Glu-PG is converted to plasmin, which subsequently hydrolyzes Bz-Arg-OEt. The amount of Bz-Arg-OH produced is recorded as a function of time and analyzed to give the corresponding rate of plasmin formation [1]. According to our data (unpublished), 1 mol of UK_h, UK_l, and UK_t contains approximately 5.5×10^{12}, 7.3×10^{12}, and 7.0×10^{12} IU, respectively. In all kinetic experiments the buffer used was 0.05 mol/l Tris-HCl, 0.1 mol/l NaCl, pH 7.8.

Results and Discussion

In table I, the calculated kinetic parameters of highly purified UK preparation are shown. The apparent dissociation constants, K_m of UK_h and UK_l were similar (31 ± 8 and 38 ± 6 μ*M*) while the catalytic rate constant, k_c, of UK_h was approximately 3 times greater than that of UK_l. All K_m values are comparable to, but the k_c values of UK_h are

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Table I. Kinetic parameters of three different UKs for Glu-PG activation

Enzyme	Catalytic constant (k_c) s^{-1}	Apparent Michaelis constant ($K_{m,app.}$) μM	Overall rate constant ($k_c/K_{m,app.}$) $M^{-1}\,s^{-1}$
UK_h, molecular weight 53,000	$k_h = 2.40 \pm 0.2$	$K_h = 31 \pm 8$	7.7×10^4
UK_l, molecular weight 33,000	$k_l = 0.83 \pm 0.10$	$K_l = 38 \pm 6$	2.2×10^4
UK_t, molecular weight 36,000	$k_t = 0.91 \pm 0.18$	$K_t = 38 \pm 4$	2.4×10^4

Values were obtained from four determinations for each example.

Discussion

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f) while the catalytic rate con-
k_h was approximately 3 times
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but the k_c values of UK_h are considerably higher than those of *Christensen* [2] ($K_m = 32 \pm 11\ \mu M$, $k_c = 0.26 \pm 0.07\ s^{-1}$). This may be due to the difference in UK molecules used; Christensen used a crude preparation (10,000 Ploug units/mg) and the molecular variation was not defined. It is now generally accepted that UK_h with a molecular weight of about 53,000 is the native form, and UK_l with a molecular weight of about 33,000 is the enzymatically degraded form of UK_h [9, 10, 12, 13]. Recently, UK_h has been shown to have two polypeptide chains of a molecular weight of 33,000 (heavy chain) and 20,000 (light chain), linked by disulfide bonds [12, 16]. The active histidine serine residue has been found to be located in the heavy chain' [12, 17]. On the other hand, UK_l has been proven to be a single polypeptide chain by SDS polyacrylamide gel electrophoresis [12, 16]. Despite the many models that have been proposed, the mechanism of the molecular change of UK_h to UK_l either during purification or in vivo, is still not completely understood. In 1967, *Lesuk* et al. [11] first demonstrated the enzymatic degradation of UK_h in purified systems, and isolated the active molecule, UK_t. However, no studies of UK_t have been reported since.

As can be seen in table I, all the kinetic parameters of UK_t were very similar to those of UK_l. From the overall second order rate constant, k_c/K_m (the fourth column), it may be deduced that the enzymatic efficiency of UK_h in native PG activation is more than 3 times greater than that in the degraded forms. From the present results and the molecular structures of UK, it can be speculated that the 'light chain' of UK_h plays a very important role in the activation step of Glu-PG to plasmin.

In recent years, several UK preparations have not only been widely used in the therapy of thromboembolic diseases, but also for enhancement of carcinostatic treatment of malignant tumors. Native UK may prove to be more effective than degraded UKs in such therapy.

References

1 Christensen, U.; Müllertz, S.: Kinetic studies of urokinase-catalyzed conversion of NH_2-terminal lysine, plasminogen to plasmin. Biochim. biophys. Acta *480:* 275–281 (1977).

2 Christensen, U.: Kinetic studies of the urokinase-catalyzed conversion of NH_2-terminal glutamic acid plasminogen to plasmin. Biochim. biophys. Acta *481:* 638–647 (1977).

3 Kitamura, M.; Chikamori, K.: Thrombolytic effect of commercial urokinase (Japanese). Yakkyoku *25:* 1581–1584 (1974).

4 Samama, M.; Cazenave, B.; Otero, A.M.: Urokinase I and II activity. Thromb. Haemostasis *40:* 578–580 (1979).
5 Lormeau, J.C.; Coulay, J.; Vairel, E.G.; Choay, J.: A comment on the activities of high and low molecular weight urokinase: fibrinolysis: current fundamental and clinical concepts, pp. 77–82 (Academic Press, New York 1978).
6 Sumi, H.; Sasaki, K.; Muramatu, M.: A simple and rapid purification method of urokinase using a [N^{α}-(ε-aminocaproyl)-*DL*-homoarginine hexylester]-Sepharose column. Acta haemat. jap. *41:* 766–770 (1978).
7 Sumi, H.; Takada, Y.; Takada, A.: The effects of metal ions on esterase activities of urokinase. Thromb. Diath. haemorrh. *39:* 46–52 (1978).
8 Weber, I.; Osborn, M.: The reliability of molecular weight determinations by dodecylsulfate-polyacrylamide gel electrophoresis. J. biol. Chem. *244:* 4406–4412 (1969).
9 Lesuk, A.; Terminiello, L.; Traver, J.H.: Crystalline human urokinase: some properties. Science *174:* 880–882 (1965).
10 White, W.F.; Barlow, G.H.; Mozen, M.M.: The isolation and characterization of plasminogen activators (urokinase) from human urine. Biochemistry, N.Y. *5:* 2160–2169 (1966).
11 Lesuk, A.; Terminiello, L.; Traver, J.H.; Groff, J.L.: Biochemical and biophysical studies of human urokinase. Thromb. Diath. haemorrh. *18:* 293–294 (1967).
12 Johnson, A.J.; Soberano, M.; Ong, E.B.; Levy, M.; Schoellmann, G.: Urinary urokinase. Two molecular or one? Thrombosis and urokinase, pp. 59–67 (Academic Press, New York 1977).
13 Alkjaersig, N.; Fletcher, A.: Metabolism of urokinase. Thrombosis and urokinase, pp. 129–141 (Academic Press, New York 1977).
14 Andrews, P.: The gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J. *96:* 595–606 (1965).
15 Gros, C.; Labouesse, P.: Study of the dansylation reaction of amino acids, peptides, and proteins. Eur. J. Biochem. *7:* 463–470 (1969).
16 Holmberg, L.; Bladh, B.; Åstedt, B.: Purification of urokinase by affinity chromatography. Biochim. biophys. Acta *445:* 215–222 (1976).
17 Ong, E.B.; Johnson, A.J.; Schoellmann, G.: Identification of an active site histidine in urokinase. Biochim. biophys. Acta *429:* 252–257 (1976).

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